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Semiautomatic Method for the Separation and Determination of Total Triiodothyronine and Thyroxine in Serum

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A semiautomatic procedure is presented for the separate determination of total T_3 and T_4 in serum in approximately 10 hrs simultaneously in 25 samples. Removal of the thyroid hormones from the serum, separation of T_3 from T_4 and concentration of the peaks were achieved in a closed system, consisting of three consecutive columns. The automatic microchemical iodine determination of T_3 and T_4 respectively, had a sensivity of less than 0.5 ng. Recovery for T_3 was 87% and for T_4 88%. High accuracy and reproducibility could be shown with pooled control serum. The overspill of T_4 into T_3 was less than 0.1% of total T_4.
The means of total serum T_3 and T_4 were 1.46 ± 0.20 (S. D.) µg/l and 76.5 ± 10.7 µg/l respectively for euthyroid controls. In patients with nontoxic goiter the T_4 levels were significantly lower than in euthyroid controls, whereas the T_3 levels and the T_3:T_4 ratio were significantly higher.

Es wird eine semiautomatische Methode für die simultane Analyse von 25 Proben beschrieben, welche die getrennte Bestimmung des Gesamt-Trijodthyronins und -Thyroxins im Serum innerhalb von etwa 10 Stunden erlaubt. Die Abtrennung der Schilddrüsenhormone von den Serumproteinen, die Trennung von T_3 und T_4 und die Konzentrierung der jeweiligen Eluate erfolgte in einem geschlossenen System, bestehend aus drei aufeinanderfolgenden Säulen. Die automatische mikrochemische Jodbestimmung für T_3 bzw. T_4 hat eine Empfindlichkeit von weniger als 0,5 ng. Die Wiederfinderaten waren für T_3: 87% und für T_4: 88%. Die Richtigkeit und Präzision der Methode waren zufriedenstellend. Weniger als 0,1% des gesamten T_4 gehen in die T_3-Fraktion ein.
Bei euthyreoten Kontrollpersonen fanden sich Serum-T_3-Spiegel von 1,46 ± 0,20 µg/l ($\bar{x}$ ± s) und Thyroxin-Spiegel von 76,5 ± 10,7 µg/l. Bei Patienten mit blander Struma waren die Gesamt-Thyroxin-Spiegel signifikant im Vergleich zu Kontrollpersonen erniedrigt, während die T_3-Spiegel und der T_3:T_4-Quotient signifikant erhöht waren.

The clinical significance of the levels of total triiodothyronine[2]) in serum is of growing interest. Several techniques for the determination of triiodothyronine in serum have been developed. Sterling and Werner have both employed paper chromatography (1—7), whereas recently radioimmunological methods have been developed (8—10). All these techniques have been subject to substantial criticism (4—7).
The method developed in our laboratory during the past years consists basically of three steps: (1) Removal of thyroid hormones from serum proteins, (2) separation of T_3 from T_4 and (3) quantitation of T_3 and T_4 by microchemical iodine determination. Continuous chromatography in a closed system consisting of three consecutive columns diminished artefacts. Simultaneous chromatography of 25 samples was achieved using a 25-channel peristaltic pump in order to obtain partial automation and standardization of the procedure.
Preliminary results of the study have been reported (11, 12).

Materials and Methods

Cation exchange resins: AG 50 W—X 4, minus 400 mesh, hydrogen form; Bio-Rex 70, 50—100 mesh, sodium form (Bio-Rad Laboratories, Richmond, Calif.). Dextran gels: Sephadex G-25, superfine; Sephadex LH-20 (Pharmacia, Uppsala).

Radioactive compounds: Liothyronine-^{125}I (3, 5, 3′-triiodo-L-thyronine), 20—50 mC/mg; L-thyroxine-^{125}I, 20—50 mC/mg; 3,5-diido-L-tyrosine-^{125}I, 25—100 mC/mg and 3-iodo-L-tyrosine-^{125}I, 0.1—2 mC/mg, all in 50% (v/v) aqueous propylene glycol solution (Radiochemical Centre, Amersham).
Stable compounds: 3,3′,5-triiodo-L-thyronine, puriss.; L-thyroxine, purum; 3,5-diiodo-L-tyrosine, purum and 3-iodo-L-tyrosine, purum (Fluka AG, Buchs, Switzerland); 3, 5, 3′, 5′-tetraiodothyroacetic acid (Serva, Heidelberg).

Removal of T_3 and T_4 from serum proteins

The cation exchange resin AG 50 W-X4 (H^+-form) was washed three times with 0.1N HCl. After discarding the last supernatant, an equal volume of 0.1N HCl was added to the washed resin. Two ml of this 1:1 (v/v) continuously stirred suspension were pipetted into each of 25 glass microcolumns (volume 5 ml) with the outlet closed (Fig. 1, a). Serum (2.5 ml) or standard solution were added. The microcolumns were then closed by glass stoppers. Removal of T_3 and T_4 from serum was obtained by mixing the content of each of the 25 microcolumns on a rotor for 30 min at room temperature. Each microcolumn was washed with 10 ml 1N potassium chloride (Fig. 1, b), to exchange all the H^+-ions against K^+-ions. The eluates containing proteins and iodide were discarded. The microcolumns with T_3 and T_4 bound to the resin were then linked to the second columns.

Separation of T_3 and T_4 on Sephadex G-25

Twentyfive glass columns (h = 130 mm, d = 11 mm) mounted in a waterbath of 27° C were filled with a suspension of 4 g Sephadex G-25 superfine and washed with 0.05M potassium phosphate buffer (pH 11.9) at a constant rate of 0.42 ml per min using a peristaltic pump (25-channel micro-pump, Ismatec, Zürich). The supernatant buffer was removed from the Sephadex. The microcolumns of the first step were then put on top of these Sephadex columns (Fig. 1, c). T_3 and T_4 were eluted together in a narrow peak from the resin with 0.05M potassium phosphate buffer pH 11.9 (peristaltic pump) and simultaneously layered onto the Sephadex

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[2]) Abbreviations: T_3 = triiodothyronine, T_4 = thyroxine, MIT = monoiodotyrosine, DIT = diiodotyrosine, PBI = protein bound iodine.

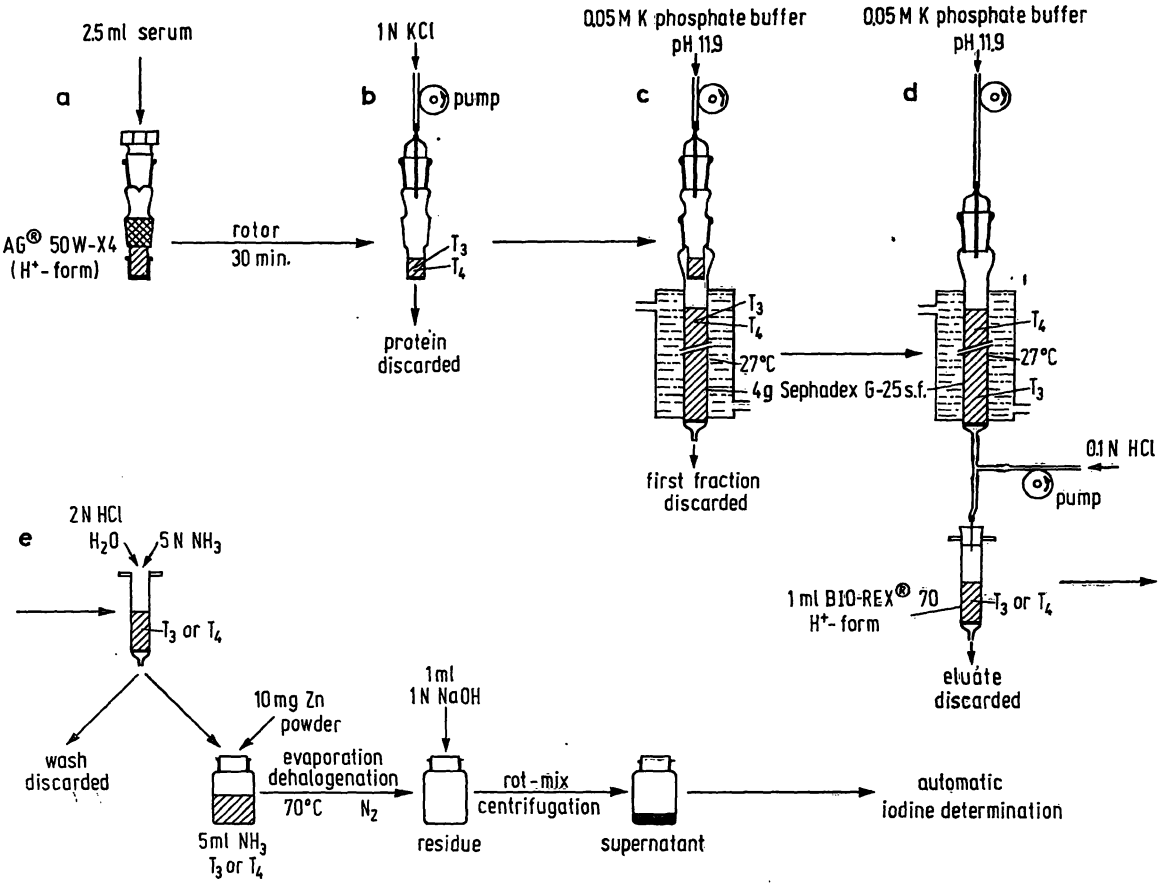

Fig. 1
Schematic presentation of the method. For explanation see text

columns. One milliliter fractions were collected and good separation could be achieved (Fig. 2). Therefore for routine purposes batch sampling of T_3 and T_4, combined with separate concentration of T_3 and T_4 on cation exchange resins, was performed (Fig. 1, d).

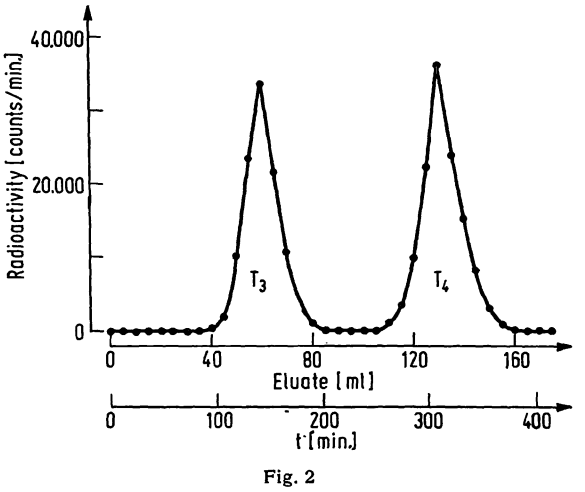

Fig. 2
Separation of T_3 from T_4 on Sephadex G-25 superfine
The figure shows the elution of radioactive standards in serum subjected to the first two steps of the method. The radioactivity of the fractions collected is given versus the volume and the time of elution respectively

Concentration of the T_3 and T_4 batches on cation exchange resin

The concentrating columns (polypropylene syringes, 2 ml) each contained 1 ml Bio-Rex 70, H^+-form. The latter was prepared from the sodium form by repeated washing with hydrochloric acid.

The first 40 ml from the Sephadex G-25 column were discarded (Fig. 2). The eluate (T_3 or T_4) was then continuously acidified with 0.1N HCl by means of a second 25-channel micro-pump (Ismatec, Zürich) at a rate of 0.23 ml per min. The fractions containing T_3 or T_4 (Fig. 2) were subsequently layered onto Bio-Rex 70 columns for retention of T_3 and T_4 respectively (Fig. 1, d).

The Bio-Rex columns containing T_3 or T_4 were first washed with 7.5 ml 2N HCl and then with 15 ml water (Fig. 1, e). T_3 or T_4 were then eluted with 5 ml 5N NH_3 into glass vials. After the addition of approximately 10 mg of zinc powder activated by cuprous sulfate for reductive dehalogenation (13), the samples were evaporated at 70° C within 30 min under nitrogen (Fig. 1, e), the gas being recycled in a closed system. This step also provided the necessary deiodination of the thyroid hormones (13). The evaporated residue was dissolved in 1 ml 1N NaOH, mixed for 20 min and centrifuged briefly.

Quantitation of T_3 and T_4

The automatic microchemical iodine determination in the solved residues was performed in a continuous flow system as published (13). The sensitivity of the microchemical analysis can be seen from the standard curve (Fig. 3). — The measured values were corrected against the yield of a control serum incubated with labeled T_3 and T_4 (Table 1). All data reported represent the mean of duplicate analyses.

Table 1
Quality control of the complete procedure

Quality control from day to day:	T_3	T_4
Recovery of added ^{125}I-labeled hormone	86.7 ± 4.9 % (N = 15)	88.4 ± 4.1 % (N = 15)
Reproducibility of control serum	1.45 ± 0.14 µg/l (N = 14)	55.8 ± 3.8 µg/l (N = 16)
Values of healthy controls: No goiter, no medication, normal weight	1.46 ± 0.20 µg/l (N = 23)	76.5 ± 10.7 µg/l (N = 23)
	Means ± SD	

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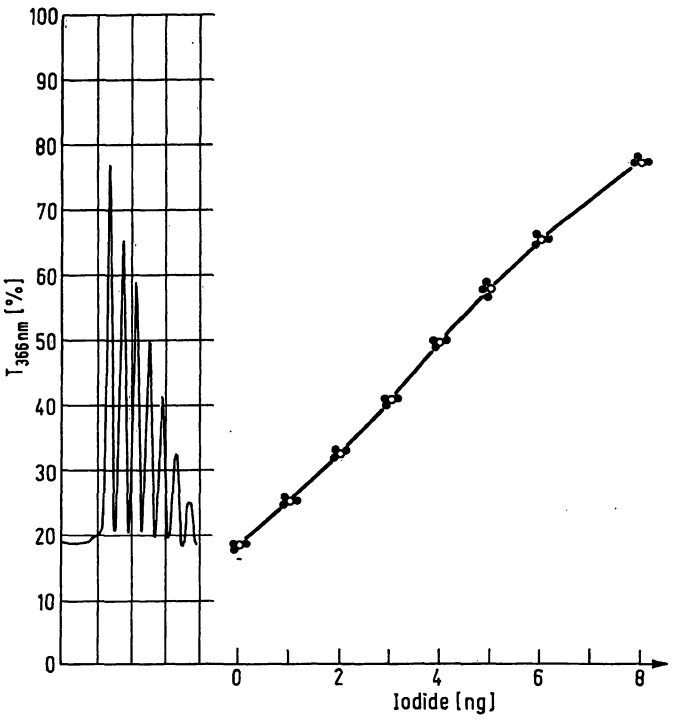

Fig. 3
Standard curve of the microchemical iodine determination
On the left is shown the automatic recording of the standards. Open circles (O) are means of triplicate determinations (●)

Results

The different steps of the method

On the first column the T_3 and T_4 content of serum was completely retained, as demonstrated by experiments with radioactive T_3 and T_4. From this first column 94% of radioactive T_3 and T_4 could be concomitantly eluted in a volume of 8 ml using continuous chromatography. Since the eluate of the first column was simultaneously layered onto the second column, no intermediate concentration step was necessary. Continuation of the chromatography in this closed system with the same buffer separated T_3 from T_4. After the elution from the second column, the radioactive T_3 could be completely recovered in approximately 45 ml (Fig. 2). Complete recovery of the T_4 was obtained in approximately 60 ml, separated from the T_3 peak by an interval of 20 ml free of radioactivity (Fig. 2).

Prior to quantitation, the eluates containing T_3 or T_4 had to be reduced in volume. Continuous acidification of the eluates from the Sephadex columns (Fig. 1, d) in the closed system used, was necessary for complete retention of T_3 and T_4 respectively on two separate cation exchange resin columns. For the final elution of T_3 or T_4 from the Bio-Rex columns a yield of 95% of the radioactivity in 5 ml of 5N NH_3 was obtained.

The sensitivity of the microchemical iodine determination (13) permitted the measurement of 0.5 ng iodine from thyroid hormones (Fig. 3) after subtraction of the blank. The introduction of the reductive dehalogenation step made it possible to estimate the iodine content of the thyroid hormones by a standard curve of iodide. The yield was 96% for cold hormones (13).

The blank value for the whole procedure was about 1 ng iodine, mainly due to the concentrating step on the Bio-Rex column. Recently, we therefore used Sephadex LH-20 instead of Bio-Rex 70. From 1 ml Sephadex LH-20 columns T_3 or T_4 were completely eluted in 3 ml solution of 15N NH_3: 50% (v/v) aqueous methanol = 1:1, resulting in blank values of less than 0.5 ng.

Quality control

The quality control of the complete procedure (Fig. 1) for the separate determination of T_3 and T_4 is presented in Table 1. The recovery of added radioactive thyroid hormones was checked from day to day and was 87% for T_3 and 88% for T_4. The recovery of cold T_3 was studied by the following experiment. Three nanograms T_3 were added to 2.0 ml control serum, the latter containing 3.26 ± 0.21 ng T_3 ($N = 6$). After the whole procedure 5.90 ± 0.33 ng T_3 ($N = 4$) were recovered, which is equal to 88% of the originally added T_3.

The precision of the method was determined from day to day with a control serum. The coefficient of variation was less than 10% for T_3 and 7% for T_4 (Table 1). The precision within a single assay was even better, namely 6.5% for T_3 and 2.7% for T_4 (12).

Specifity

The following rechromatography experiment was performed to check the overspill of T_4 into the T_3 peak (Table 2, Fig. 2):

Radioactive standards of T_3 and T_4 were incubated each with 2 ml control serum and, after the whole separation procedure (Fig. 1, a—d), the peaks of the T_3 and T_4 area were concentrated on AG 50 W-X4 instead of Bio-Rex 70 as the third columns. These columns were used as the first columns of the rechromatography through the whole procedure (Fig. 1, a—d). The results are shown in Table 2. The radioactive standards used were contaminated with 2.1% iodide (T_3) and 1.6% (T_4) respectively. The overall yield was 91.7% for T_3 and 92.9% for T_4 after rechromatography. The radioactive contamination of the T_3 standard found in the T_4 area was 0.99% after the first chromatography, 0.25% after the second chromatography. These radioactivities were probably due

Table 2
Rechromatography of radioactive T_3 and T_4 standards
For technical details and explanation see text

	I^-	T_3	T_4
T_3-^{125}I: 1st chromatography	2.1%	↓	0.99%
2nd chromatography	—	91.7%	0.25%
T_4-^{125}I: 1st chromatography	1.6%	↓	↓
2nd chromatography of T_3 in T_4 standard	—	1.31%	↓
2nd chromatography of T_4	—	0.09%	92.9%

to tailing of the T_3 peak and not due to real T_4 contamination.

The T_4 standard was contaminated with 1.31% of radioactivity which was identified as T_3 in the second chromatography (Table 2). Rechromatography of the T_4 peak showed only a very small amount of T_3 (0.09%). Thus, the overspill of T_4 into T_3 was less than 0.1% of total T_4. — Further, 350 ng T_4 of a freshly prepared cold standard solution were applied to the procedure without prior chromatographic purification. No measurable T_3 was found.

The iodide content of serum is eluted from the first column (Fig. 1) and discarded together with the serum proteins. — The MIT and DIT fractions which are eluted from the first column onto the second column appear before the T_3 peak, as proved with radioactive standards.

Added inactive tetraiodothyroacetic acid (tetrac) was eluted together with T_4. — When a serum heavily contaminated with exogenous iodine (e. g. X-ray contrast media), as shown by excessively high $PB^{127}I$ values, was subjected to the procedure, some overspill of the contaminant into the T_3 area was observed.

Euthyroid controls and nontoxic goiter patients

The normal ranges of the total serum T_3 and T_4 contents calculated from the 23 euthyroid controls studied so far are given in Table 1. The T_3:T_4 ratio in serum was 1:44 on a molar basis.

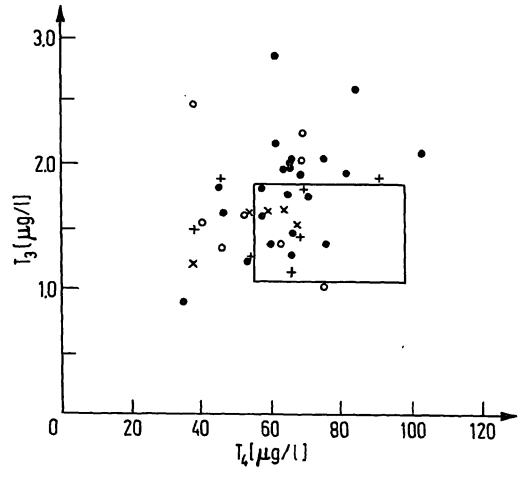

Fig. 4
Individual serum T_3 levels versus T_4 levels of patients with nontoxic goiter in comparison with the normal range of euthyroid controls
The rectangle represents the normal range (means ± 2 SD) of T_3 and T_4 of euthyroid controls. Diffuse nontoxic goiter (●, N = 24), nodular nontoxic goiter (+, N = 7), recurrent diffuse nontoxic goiter (O, N = 7) and recurrent nodular nontoxic goiter (×, N = 5)

Figure 4 shows the normal ranges of T_3 and T_4 of euthyroid controls and the individual T_3 and T_4 levels of patients with diffuse or nodular nontoxic goiter. The individual values of T_4 of the goiter patients are in the lower half of the normal range or just below. In contrast, the T_3 levels are normal or slightly elevated. The same was found for recurrent nontoxic goiter. The means of the serum levels of patients (N = 43) with nontoxic goiter were significantly elevated for T_3: 1.71 ± 0.41 (S. D.) µg/l compared with euthyroid controls (1.46 ± 0.20 µg/l, N = 23, $p < 0.005$). The T_4 values were significantly diminished: 61.9 ± 14.2 µg/l versus 76.5 ± 10.7 µg/l ($p < 0.0005$). The T_3:T_4 ratio (ng:µg) of nontoxic goiter patients was therefore significantly higher than of euthyroid controls: 28.8 ± 8.9 versus 19.6 ± 4.1 ($p < 0.0005$).

Discussion

The *normal range* of the *total* T_3-level in serum (1.06—1.86 µg/l) obtained by this method is obviously lower than the normal ranges reported by other authors (1—4, 7). This discrepancy is probably caused by the use of different methods. The two main sources for excessively high T_3 levels are: (1) T_4 overspill into T_3 and (2) artefacts arising from chromatography. Paper chromatographic techniques have recently been seriously questioned in this respect (4—7).

The *method* presented here has the following *advantages*:

1. Column chromatography is used throughout the method without intermediary organic solvent extraction or volume reduction steps before the separation of T_3 from T_4. This diminishes the chance of chromatographic artefacts, for example ester formation (4, 5, 7). In addition this approach permitted simultaneous chromatography of 25 samples.

2. The automation of the chromatography on three consecutive columns (closed system) and of the iodine determination (13) obviously resulted in sufficient standardization of the yields (average 87%). As a consequence the addition of internal radioactive standards to all samples could be omitted. Internal radioactive standards are probably sources for overcorrection (6). This might be of major importance in methods with yields of around 50%, especially when the radioactive standards were contaminated or had low specific activities.

3. The chromatographic system used separates T_3 from T_4 on the basis of different pK values of the phenolic groups of the molecules (6.73 for T_4 and 8.45 for T_3) (14). T_3 is eluted before T_4 with an overspill of less than 0.1% of total T_4 into T_3. Therefore the error in the T_3 determination due to T_4 contamination is less than 5%.

Our results for T_3 levels are in good agreement with the recently published data based on radioimmunological determination (15).

The serum T_4 levels of patients with *nontoxic goiter* tend to be low-normal, the serum T_3 levels to be normal or slightly elevated. The patients are from an area of iodine deficiency with urinary iodine excretions of approximately 40 µg/day (unpublished data). Nontoxic goiter patients of our area were earlier shown to have slightly diminished values of PBI and T_3 – uptake as compared with euthyroid controls (16, 17). The same

was observed for the group of patients studied here. The normal or even elevated total T_3 levels could be interpreted as an attempt to preserve euthyroidism in the patients with endemic goiter. Determination of TSH levels in our patients will show, if the increment of T_3 in relation to T_4 is a consequence of elevated TSH levels, as postulated by some authors (17, 18) and questioned by others (19, 20). T_3 preference could also be interpreted on the basis of substrate deficiency (iodide) for thyroid hormonogenesis (21).

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